

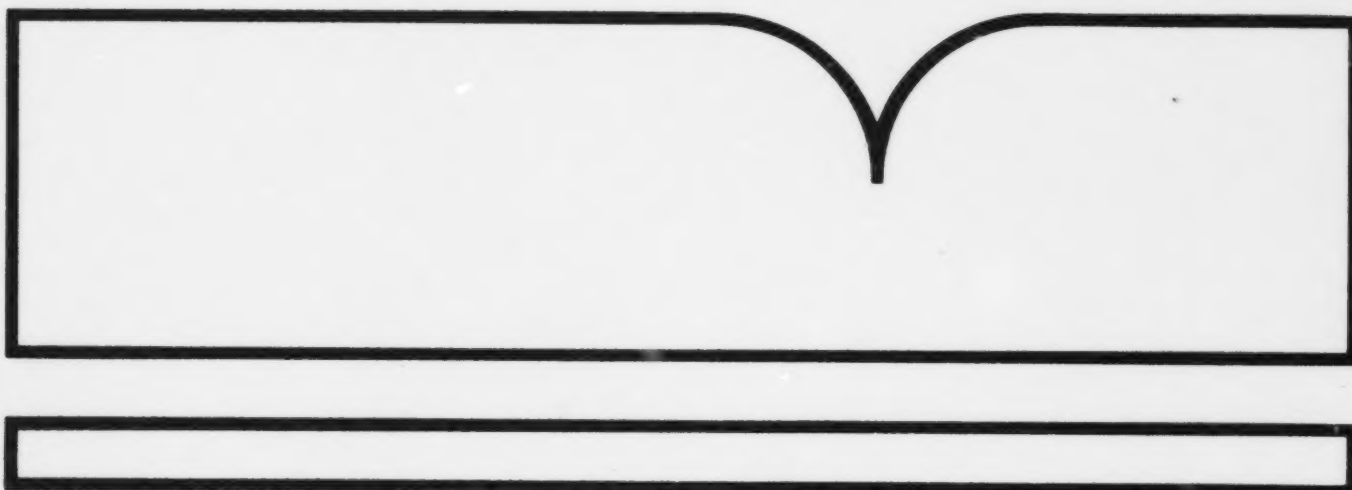
Growth Characteristics, Morphology, and
Phospholipid Composition of Human Type 2
Pulmonary Alveolar Cells Grown in a
Collagen-Free Microenvironment

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16. ABSTRACT Human lung epithelial cells have been cultured and characterized for phospholipid content. Any residual fibroblasts were removed by selective trypsinization within the first 48 hours in culture. Epithelial cells were serially subpassaged when cultures reached ca. 80% confluency. Electron microscopic evaluation of both monolayers and cell suspensions showed cultures to be composed of a single cell type. These cells had a microvillar free surface and were attached by desmosomes which characterized them as epithelial. They contained lamellar inclusions which is typical of type II cells. Fetal lung (age 18-20 weeks) cell suspension processed for electron microscopy prior to culturing showed cells to be undifferentiated, epithelial-like with small microvilli along cell borders and desmosomes at cell cultured epithelial cell deonstrated that the lamellar inclusions were not observed in these cells. Ultrastructural studies of the cultured epithelial cell demonstrated that the lamellar inclusions had a slightly positive reaction when tested for acid phosphatase. Phospholipid analysis of these lung epithelial cells showed a phsopholipid composition consistent with that found in surfactant-containing type II cells. Cultured epithelial cells stained with phosphine 3-R demonstrated a green fluorescent cytoplasm and nucleus with brightly fluorescent yellow orange perinuclear particles. The preceding characterization of these cells leads us to conclude that they exhibit structural and biochemical features commensurate with type II epithelial cells from human lung					
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GROWTH CHARACTERISTICS, MORPHOLOGY, AND PHOSPHOLIPID COMPOSITION OF HUMAN TYPE II PULMONARY ALVEOLAR CELLS GROWN IN A COLLAGEN-FREE MICROENVIRONMENT

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SUMMARY

Human lung epithelial cells have been isolated and maintained in pure culture and characterized during their time in culture. Any residual fibroblasts were removed by selective trypsinization within the first 48 h in culture and the residual epithelial cells from the primary culture grew to confluent density. The epithelial cells at Passage 2 or greater were serially subpassaged when cultures reached ca. 80% confluency. This procedure permitted us to conduct biochemical and structural studies of starting materials and subsequent population doublings.

Electron microscope evaluation of both initial monolayers and cell suspensions showed cultures to be composed of a single cell type. These cells had microvilli on their free or apical surface. Subsequent population doubling level 1 up to 5 exhibited the same structures. They contained lamellar inclusions, which are typical of Type II alveolar epithelial cells. Fetal lung (age 18 to 20 wk) cell suspensions processed for electron microscopy before culturing showed cells to be undifferentiated, epithelial-like with small microvilli along cell borders, and with desmosomes at cell junctions. Lamellar inclusions were not observed in these cells. Ultrastructural studies of the cultured epithelial cells demonstrated that the lamellar inclusions had a slightly positive reaction when tested for acid phosphatase. Phospholipid analysis of these lung epithelial cells showed a phospholipid composition consistent with that found in surfactant-containing Type II cells. Cultured epithelial cells stained with phosphine 3-R demonstrated a green fluorescent cytoplasm and nucleus with brightly fluorescent yellow-orange perinuclear particles. The preceding characterization of these cells leads us to conclude that they exhibit structural and biochemical features commensurate with Type II epithelial cells from human lung. Moreover, these selection techniques applied to the isolation of human lung Type II cells from the tissue permit us to study the differentiative function of these cells routinely under conditions of growth in vitro.

Key words: Type II lung cells; phospholipid; human lung fibroblasts.

INTRODUCTION

Mammalian alveolar Type II lung cells have been grown successfully in vitro (1-8). More

recently, methods have been developed for culturing human mixed cell cultures containing pulmonary Type II alveolar cells using both lung explants (9) and aggregate lung cell suspensions (2,10). Seeding of cells onto a collagen substratum was suggested in order to establish organotypic primary epithelial cell populations and to eliminate contaminating fibroblasts. These were aggregate

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cell cultures containing up to 2×10^6 cells/culture (3,11). However, these populations could not be serially subpassaged. These cells do not exhibit the same growth kinetics on the collagen substratum as keratinocytes. In another system where a collagen substratum was initially required for culturing human skin epithelial cells (12,13), techniques were soon developed that allowed the growth and proliferation of human epidermal skin cells on a plastic substratum free from contaminating fibroblasts and without the addition of collagen (12-14). To eliminate any effect the substratum would have on altering the biochemistry of the cell, when epithelial cells were grown on a collagen substratum and fibroblasts isolated from the same tissues would not grow on collagen, we initiated studies to grow lung epithelial cells on a plastic substratum.

In this report we describe a culturing procedure that permits the growth of single cell suspensions of human Type II lung epithelial cells *in vitro* on a plastic collagen-free substratum and free of contaminating fibroblasts. The *in vitro* growth characteristics of these cells as well as their morphology, histochemistry, and phospholipid composition are detailed. The technique has been presented in preliminary form and represents an outgrowth of our earlier studies with differentiating keratinocytes from human foreskin (12,14).

MATERIALS AND METHODS

Establishing primary cultures. Primary cultures of lung epithelial cells were established from human fetal lung tissues (18 to 20 wk) obtained by surgical excision (2). Growth medium, minimal essential medium (MEM), was prepared as follows: Eagle's minimal essential medium (IX, GIBCO, Grand Island, NY), containing Hanks' balanced salt solution (HBSS) and 25 mM HEPES buffer at pH 7.2, was supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM glutamine (M.A. Bioproducts, Walkersville, MD), 0.2% sodium bicarbonate, and 50 μ g/ml gentamicin sulfate (Gentocin, Schering Corp., Kenilworth, NJ).

The best cell growth was obtained when the tissue was used immediately after surgery. However, lung tissue can be stored in MEM supplemented only with 5% fetal bovine serum (FBS) for 2 wk at 12° C if the medium is changed every 2 d.

Lung tissue, cut into 1 cm² pieces, was extensively washed with MEM only and then was minced into ca. 1 mm pieces in four-well tissue

culture plates (26 cm², no. FB-4-TC Linbro Scientific, Hamden, CT) containing 6 ml MEM and 2.5 ml 0.5% collagenase (Worthington Biochemical Corp., Freehold, NJ, Type CLS no. 4197, 115 U/mg) per well. These suspensions were then transferred to 15-ml centrifuge tubes containing an additional 6 ml of MEM supplemented with 20% FBS and incubated for 4 h at 37° C in a 4% CO₂-enriched air atmosphere. After incubation, the cell suspensions containing dissociated cells were centrifuged at 150 $\times g$ for 7 min and the pellet was resuspended in MEM-50% FBS and recentrifuged at 150 $\times g$ for 3 min. This pellet was resuspended in MEM-50% FBS and an aliquot of dispersed cells (ca. 20 000 cells/ml) was seeded into 25 cm² flasks, (Corning, Corning, NY) to give an attachment density of 10 000 cells/cm² after 2 h. At that time the residual unattached supernatant cell suspension was poured off the attached cells and incubated an additional 2 h with 5 ml of MEM-50% FBS. This procedure minimized attachment of fibroblasts. Again the attached cells were gently rinsed once and refed with 5 ml of MEM-50% FBS. Twenty-four hours later the cultures were rinsed once to remove debris and nonattached cells and refed with 5 ml of MEM-30% FBS.

Preparation of epithelial cell cultures. Twenty-four to forty-eight hours after plating, when cell colonies contained 15 to 50 cells/colony, the cultures were trypsinized to remove residual fibroblasts. Selective removal of the fibroblasts by trypsinization was carried out in the following manner: cultures were rinsed twice with MEM to wash out any residual FBS and then overlaid with 0.5 ml of 0.1% trypsin (Worthington Biochemical Corp., Freehold, NJ, lyophilized 9300 BAEE U/mg, lot TL-3BP) dissolved in calcium- and magnesium-free HBSS (HBSS minus Ca⁺² minus Mg⁺²) containing 0.02% ethylene bis-(oxyethylenitrilo) tetraacetic acid (EGTA) (Eastman Kodak, Rochester, NY). Within 30 s, the fibroblasts floated off the substratum but the epithelial colonies remained firmly attached. Enzymatic action was stopped by the addition of 5 ml MEM plus 10% FBS. Remaining fibroblasts were removed by rinsing and mechanical agitation in the presence of 5 ml MEM. Selective trypsinization was repeated at 48-h intervals if any residual fusiform-appearing fibroblasts were apparent.

Serial subpassaging of epithelial cells. Lung epithelial cells were subpassaged serially when

cultures reached 80% saturation density, Table 1. The seeding density of epithelial cells was determined by dividing the saturation density in the flask by the total area of the flask. This density occurred 3 d after residual fibroblast removal.

Cultures were rinsed with 5 ml MEM to remove FBS and then with 5 ml HBSS minus Ca^{+2} minus Mg^{+2} containing 0.02% EGTA (pH 7.2). In ca. 2 min, at room temperature, epithelial cell colonies rounded up and lifted off the substratum and dissociated into single cells. Free-floating epithelial cells were recovered by centrifugation at $150 \times g$ for 7 min. The pellet was resuspended with MEM plus 30% FBS. Cells were dispersed by pipetting them up and down in a 10 ml plastic pipette several times to obtain a uniform cell suspension. This cell suspension was seeded into 25-cm² flasks at a 8200 cells/cm² density. Subsequent subpassages were carried out in the same manner. The initial seeding of the mixed cell population was done at a cell density to yield an attachment density of 10 000 cells/cm². Serial subpassaging of the cells after establishment of the primary culture was done at 8200 cells/cm². The absolute plating efficiency was $\geq 95\%$ and the saturation density was $32\,800 \pm 80$ cells/cm². After establishing the primary culture there was a slight increase in cell size.

TABLE 1

COMPARATIVE GROWTH CHARACTERISTICS OF HEL
FIBROBLASTS AND TYPE II EPITHELIAL
CELLS IN VITRO

Cell Type ^a	Cells Seeded (1:4)/cm ²	Saturation Density/ cm ²	Life span PDL
Type II cells ^b	8 200	32 800	34 $\pm$ 5
Lung fibroblasts ^c	10 000	40 000	45 $\pm$ 7

^a These cells were companion cultures at PDL 1, prepared as described in the text, and were seeded on the plastic substratum of the flask.

^b The epithelial morphology of these cells changes to a polygonal transitional morphology after three passages (16). The value stated for saturation density was calculated from the total cell count (actual) divided by the total area of the flask.

^c The initial seeding of lung fibroblasts into a monolayer culture, following the selective removal of these cells from the mixed culture by trypsinization, at 16 000 cells/cm², yielded 50 000 cells/cm² at the first saturation density. At the first passage of the cells, seeding of the cells at a density of 10 000 cells/cm² yielded a saturation density of ca. 40 000 cells/cm².

The saturation density of the primary culture was ca. 40 000 cells/cm². The serial subpassaging of the cells at 1:4 permitted us to control the biochemical analysis at population doubling level (PDL) 2 to 4, (12).

Phospholipid analysis. For phospholipid analysis, cells from three 25-cm² flasks or one 75-cm² were scraped off with a rubber policeman into a minimal volume of Moscona's saline. Cells at PDL 4, pelleted by centrifugation at $350 \times g$ for 10 min, were extracted with 12 ml of chloroform:methanol (2:1, vol:vol). The lipid extract was washed and individual phospholipids were purified by a combination of column and thin layer chromatography (15). Lipids were quantitated by assay for phosphorous (16).

Phosphine 3-R procedure. Fetal lung epithelial cells were grown in 35-mm diam tissue culture plates for 3 to 4 d. Cells were rinsed twice with phosphate buffered saline (PBS, pH 7.4) and then 2 ml PBS containing 0.25 ml of 0.1% phosphine 3-R dye (ICN Pharmaceuticals, Inc., Plainview, NY) in 0.1% NaCl. The stock solution was filtered before use through a 1.2 μm Millipore filter under vacuum. After 60 s, the dye solution was poured off and cells were washed with PBS twice and observed under epifluorescence (Optiphot, Nikon, Garden City, NY).

Latex bead experiment. Fetal lung epithelial cells grown in 25-cm² flasks for 3 to 4 d were rinsed once with MEM and then 1 ml of a 0.01% latex bead solution (2.2 μm Dow Chemicals Co., Midland, MI) (in MEM plus 20% FBS) was added to the flask and incubated 30 min at 37° C. In other experiments, several flasks of cells were serum deprived for 48 h and then incubated for 30 min at 37° C with latex beads. These experiments were introduced to investigate the phagocytic properties of the lung epithelial cells in culture.

Preparation of monolayers for electron microscopy. Either monolayer cultures of human lung epithelial cells at Passage 1 or cell suspensions were rinsed free of medium with HBSS minus Ca^{+2} minus Mg^{+2} and fixed in 2% glutaraldehyde for 1.5 h in 0.75 M Sorenson's phosphate buffer, pH 7.4, containing 0.05 M sucrose and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 30 min at room temperature. Cells were rinsed in 0.1 M Sorenson's phosphate buffer, rinsed in 30% ethanol and stained en bloc with 2% uranyl acetate in 30% ethanol for 30 min. Cells were dehydrated and embedded in Epon 812. Sections were cut parallel to the

monolayer surface and the thin section was stained with lead citrate and uranyl acetate. One micrometer sections of the monolayers were examined by light and phase microscopy after staining with 1% basic fuchsin in 50% acetone for 10 min at 60° C or with 0.1% aqueous toluidine blue for a few seconds at room temperature, (17).

Histochemical demonstration of acid phosphatase activity. Epithelial cells from primary cultures were trypsinized off the culture flasks as described and suspended in HBSS minus Ca^{+2} minus Mg^{+2} . Cells were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, rinsed in this buffer, and placed in a Barka-Anderson (18) medium for acid phosphatase for 30 min at 37° C. After exposure to the incubation medium, cells were rinsed in 0.1 M cacodylate buffer, pelleted, and postfixed for 1 h with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, and processed for electron microscopy. In control experiments, the β -glycerophosphate was omitted from the incubation medium. For light microscopy, the monolayer cultures were fixed with cold 10% formalin, stained with naphthol AS-BI hexazonium parasoanilin, and examined at either $\times 20$ or $\times 100$.

RESULTS

Growth characteristics of lung epithelial cells and fibroblasts. Lung epithelial cells grew in discrete colonies, which merged after a few days into a distinct monolayer (Figs. 1 and 2). When cell cultures reached 80 to 90% confluency, they were serially passaged to promote optimum growth, dissociation, and continued growth (Table 1). Cells retained an epithelial morphology for at least three subpassages after the formation of monolayers in culture. Lung fibroblasts proliferated randomly until they reached confluency at which point they became whorls with a parallel orientation (Fig. 3). Fibroblasts could be trypsinized selectively and removed from cultures without seriously disturbing the epithelial monolayers. Round cytoplasmic inclusions were observed in epithelial monolayer cultures as early as Day 2 after seeding into flasks. These cytoplasmic inclusions were absent from the fibroblast cultures.

A difference in growth properties was observed between lung epithelial cells and lung fibroblasts (Table 1). Epithelial cells remained in contact with each other after cell division whereas lung fibroblasts, after going through a cycle of cell division, migrated away from each other. In



FIG. 1. Photo showing discrete colony of lung Type II cells, fixed in 3% buffered formate and stained with hematoxylin and eosin. $\times 20$.

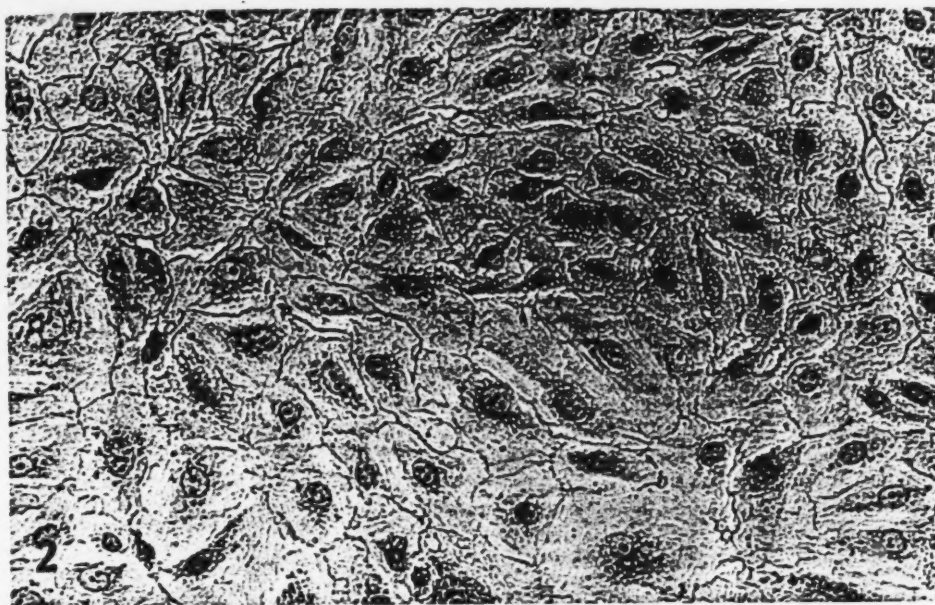


FIG. 2. Photo showing monolayer of lung Type II epithelial cells cultured, fixed in 3% buffered formate and stained with hematoxylin and eosin. $\times 100$.

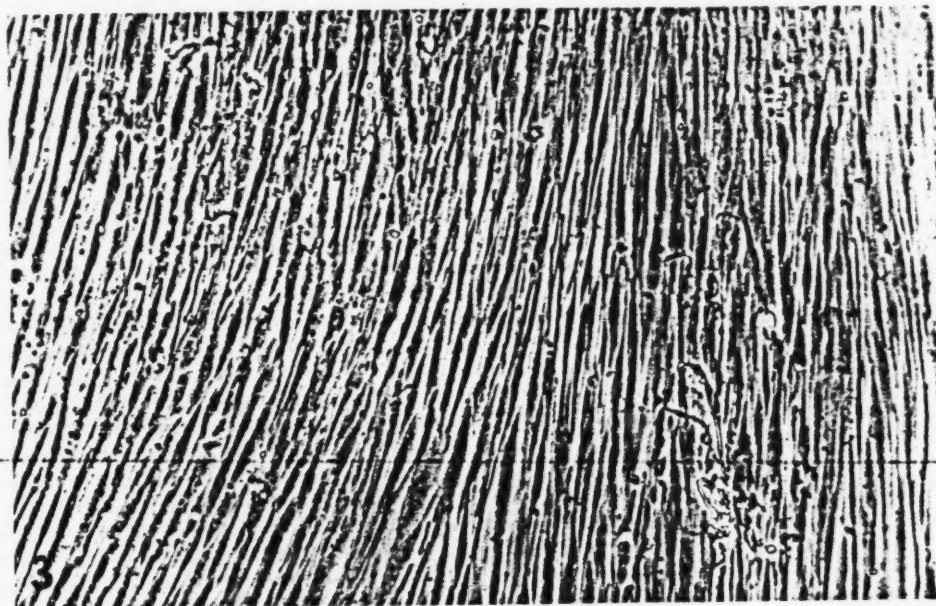


FIG. 3. Lung fibroblasts prepared from lung tissue where the lung epithelial cells were propagated in a separate flask (Fig. 1), fixed in 3% buffered formate and stained with hematoxylin and eosin. $\times 100$.

addition, epithelial cells formed a concentric colony morphology comprised of circular patterns without parallel orientation, but fibroblasts continued dividing until whorls of parallel cells were formed (3 to 4 d). It was interesting to note that population doubling times were different. As the epithelial cells were subpassaged, the doubling times increased up to 7 d compared to 3+ d for primary cultures. Populations of subpassaged lung fibroblasts reached confluency in 3 d when they split 1:4. Lung epithelial cells exhibited active proliferation throughout the colony whereas confluent dense colonies of fibroblasts exhibited active proliferation only at or near the periphery of the colony, i.e. when the fibroblasts came in contact with each other they ceased to exhibit mitotic activity; lung epithelial cells did not stop dividing but grew into a saturation dense layer. The lung epithelial cell populations grew in concentric patterns for 3 to 4 PDL before undergoing morphological transition, viz., changing from an epithelial round shaped cell to a polygon-shaped cell. These cells were truncated (had shortened morphology) and did not exhibit the typical fusiform (long, tapering toward each end) fibroblast morphology. Furthermore, these cells after 5 to 6 PDL still retained the intracytoplasmic inclusions and exhibited no junctional complexes, cell-to-cell. Epithelial cell populations had a life span of approximately 34 ± 5 PDL similar to the number of PDL observed for lung fibroblasts (45 ± 7).

Morphology of cultured lung epithelial cells. Phase and electron microscopy of lung epithelial cell monolayers indicated that the cultures were composed of a single type of epithelial-like cell. These large, round shaped or polygonal shaped cells had one and occasionally two nuclei with several prominent nucleoli (Fig. 4). The cells seemed to be polarized with respect to location of nucleus, cytoplasmic organelles, and apical border (Figs. 4 and 5). Examination of these cells by low power electron microscopy revealed that the peripheral cytoplasm was generally free of organelles and inclusions except for 5.0 to 7.0-nm filaments, which were quite abundant and extended through the cytoplasm in prominent bundles or as cytoplasmic scaffolding (Fig. 5). Filament networks and bundles were frequently noted beneath the plasma membrane of the cells (Figs. 5 and 6). Cells were joined at their borders by desmosomes that had filaments associated with them (Fig. 6). Blunted microvilli (Fig. 5) were seen along the free borders of most cells.

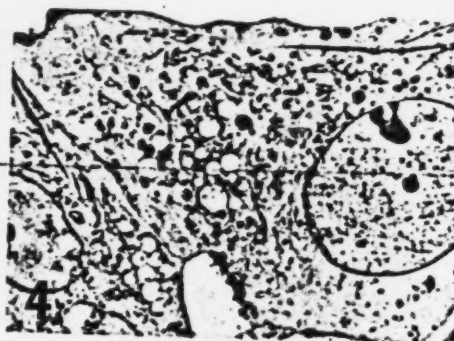


FIG. 4. Type II alveolar cell in monolayer culture. Note the contour of the cell and intimate association with adjacent epithelial cells. Clear membrane inclusion containing an ill-defined material characterize these cells. Phase contrast microscopy, stained with toluidine blue. $\times 3420$.

Cytoplasmic membrane-bound inclusions of variable size and complexity were the most conspicuous feature of the lung epithelial cells at the ultrastructural level (Figs. 5, 7, 8). These inclusions were quite variable and were composed of membrane whorls or lamellas and vesicles or vesicle groups enclosed by a single membrane (Figs. 7-9). In that morphology of the lamellar bodies varies with the fixation procedure, we have not included periodicity measurements. These variations were thought to represent different stages of development of lamellar inclusions. Vesicles present within the inclusion exhibited a matrix material of moderate to heavy electron density. The outer membrane of the inclusions was, in many instances, incomplete (Figs. 7 and 8). Small cytoplasmic smooth-surfaced vesicles, as well as elongated saccules, were in abundance near the inclusions and frequently seemed to fuse or become included within the membrane-bound inclusions and whorls (Figs. 7 and 8). In many instances, the membrane whorls were tightly packed and the complex exhibited high electron density. Vesicles in these dense inclusions were present in limited numbers of (Figs. 6 and 8). The more electron-dense inclusions were generally smaller than the more vesiculated, less organized inclusions.

The Golgi apparatus, well developed in these cells, was composed of groups of cisternae and associated with large numbers of smooth-surfaced vesicles and elongated saccules containing a material of light-to-moderate density (Fig. 9). Many of these vesicles were clustered together

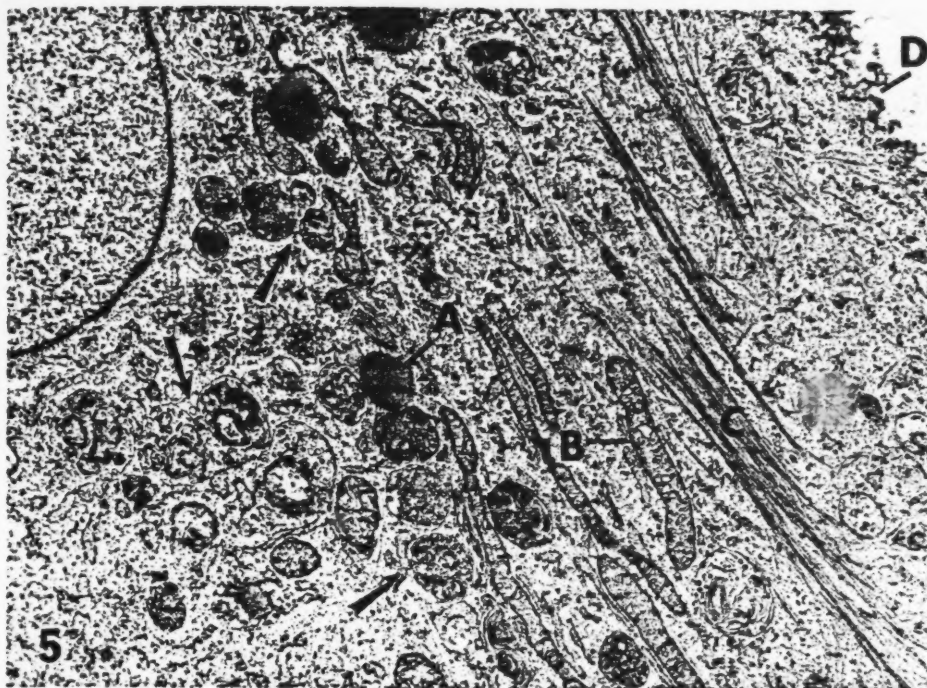


FIG. 5. Low power electron micrograph of a cultured lung epithelial cell showing membrane-bound multilamellar inclusions (arrows), several lipid droplets (A), mitochondria (B), filament bundles (C), and poorly defined microvilli (D). $\times 13\,000$.

and evidence of membrane confluency was noted. Groups of vesicles in this region were invested by a limiting membrane (Fig. 9).

Autophagic vacuoles were occasionally observed in cultured cells. Mitochondria were elongated or filamentous and were present in moderate numbers in the cytoplasm (Figs. 5, 7, and 9).

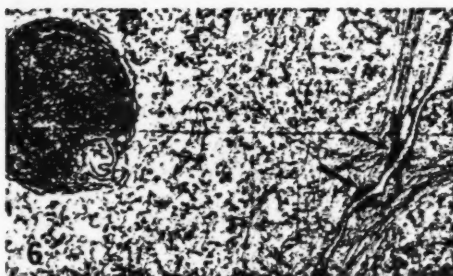


FIG. 6. Micrograph illustrates desmosomal attachment (arrows) between two epithelial cells. Cytoplasmic filaments can also be seen as well as a fully developed electron dense multilamellar inclusion. $\times 19\,444$.

Some mitochondria exhibited disrupted cristas and localized distension in this region of the organelle. The rough endoplasmic reticulum (RER) was moderately to well developed in these epithelial cells (Fig. 8). Scattered ribosomes and polysomes were found throughout the cytoplasm. Microtubules were prominent, and occasionally small, osmiophilic fat droplets (Fig. 5) were noted in the cytoplasm of cultured cells.

Ultrastructure of lung tissue prior to cell suspension and culturing. Electron microscope observation of fetal lung (18 wk gestation) showed that the parenchymal cells were basically undifferentiated and epithelial in nature. The lining cells rested on a basal lamina, had small microvilli along their apical borders, and were joined by desmosomes (Fig. 10). Mitochondria and RER were sparse but ribosomes were relatively abundant. An occasional lipid droplet was present in the cytoplasm but lamellar inclusions were not present. It is noteworthy that epithelial cell cultures derived from this fetal lung specimen developed typical lamellar cytoplasmic inclusions

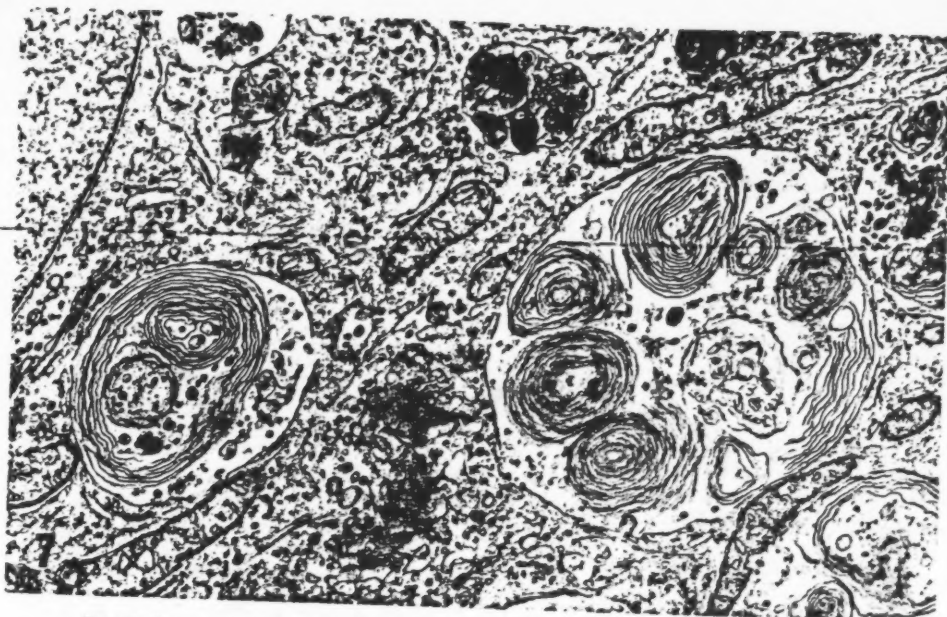


FIG. 7. Groups of multilamellar whorls and vesicles are enclosed with incomplete surrounding membranes. Many cytoplasmic vesicles are distributed in the cytoplasm and in close association with the cytoplasmic inclusions. X32 000.

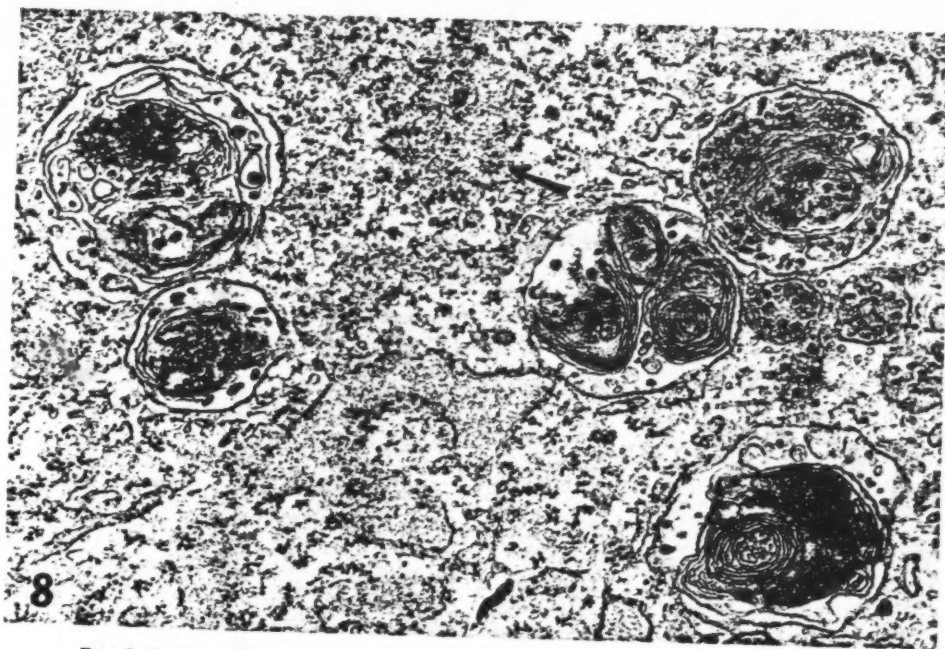


FIG. 8. Various stages in the development of the whorl-like multilamellar inclusions showing differences in membrane and vesicle compaction and electron density. Polysomes are evident in the cytoplasm and in association with the endoplasmic reticulum; the spiral arrangement of polysome on the RER is shown in tangential section (arrow). X48 000.

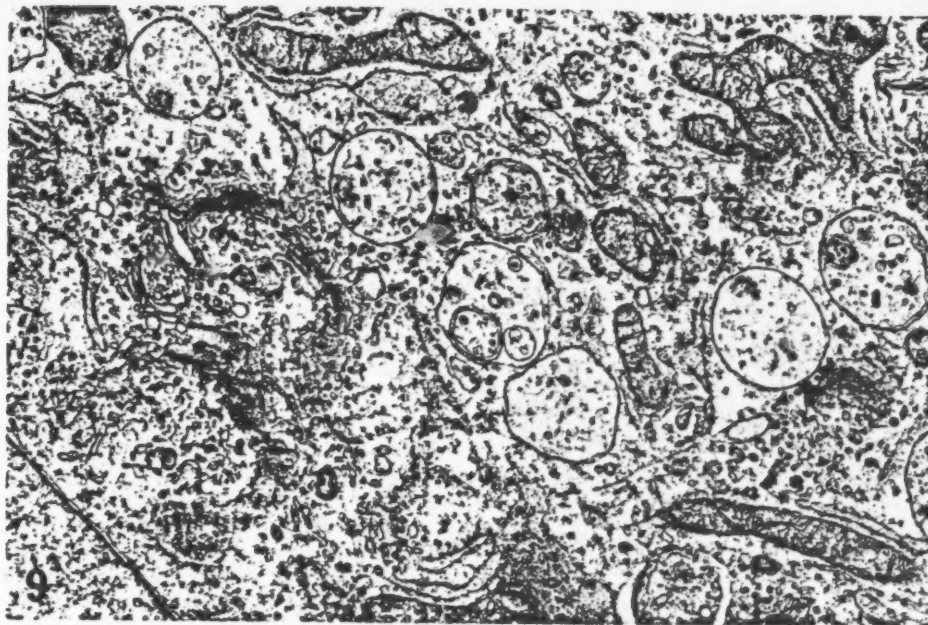


FIG. 9. A well-developed Golgi complex is illustrated as well as immature forms of the multilamellar inclusions. $\times 50\,000$.

after 2 d cultivation. It is suggested that the shock of culturing triggered the differentiation process.

Acid phosphatase reaction. Epithelial cells, grown in culture for 5 d, were tested for the presence of acid phosphatase activity, a marker enzyme found in autophagic vesicles for lysosome activity. Acid phosphatase activity was not ob-

served at the light microscope level using the naphthol AS-BI hexazonium pararosanilin staining method. However, electron micrographs of lamellar inclusions, described above, showed a positive reaction indicated by moderate electron density (Fig. 11 a and b) found along the periphery of the inclusion body.

Phosphine 3-R reaction. At Days 2, 4, and 6 after seeding of the cultures, they were then stained with phosphine 3-R dye. The cells exhibited a green fluorescent cytoplasm and nuclei (Fig. 12). The fluorescing nuclei were surrounded with fluorescing yellow-orange organelles (Fig. 12). Cultures that were in a saturation-dense stage after 2 wk, when treated with phosphine 3-R, also contained the yellow-orange cytoplasmic bodies. Cells grown 48 h without FBS and then treated with phosphine dye showed a marked increase in numbers of fluorescent inclusions as compared with those grown in the presence of FBS.

Latex bead experiment. Lung epithelial cells after 2 to 4 d in culture were evaluated for their phagocytic activity. The cells were incubated with $2\text{-}\mu\text{m}$ latex beads. Beads were seen on the surface of the cells but were not internalized

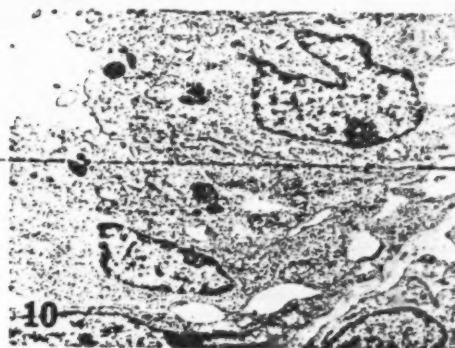


FIG. 10. Electron micrograph of lung cell suspension prior to seeding. Cells are undifferentiated and epithelial-like. $\times 7315$.

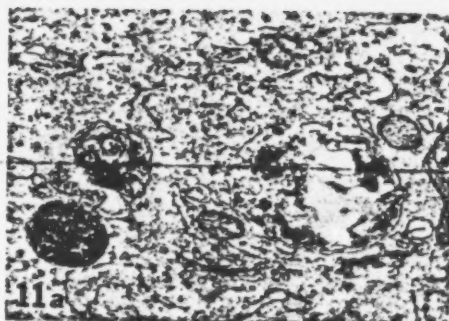


FIG. 11. Electron micrographs of lung epithelial cell showing positive reaction of lamellar inclusions to presence of acid phosphatase. a, $\times 20\,520$; b, $\times 36\,480$.

(Fig. 13). Cells grown 48 h in serum-free medium and then incubated with latex beads showed the same result.

Phospholipid characterization. The phospholipid composition of the pure culture of lung epithelial

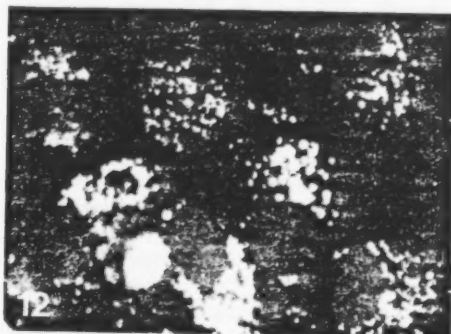


FIG. 12. Alveolar Type II cells, Day 3 in culture, showed brightly fluorescent perinuclear particles when stained with phosphine 3-R dye. $\times 308$.

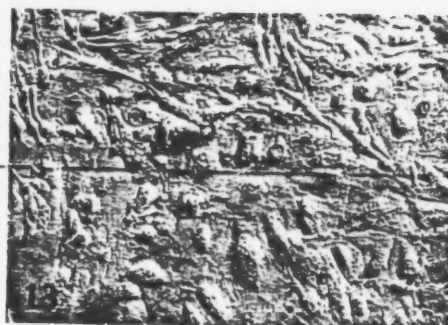


FIG. 13. Latex beads are clearly seen on surfaces of lung epithelial cells, 2 to 4 d in culture, after 30 min incubation with the $2\ \mu$ beads at 37°C . Hoffman modulation contrast. $\times 38$.

cells was measured as the cells approached confluency to determine if the cells contained surfactant indicative of Type II cells. Table 2 shows that the major species present was phosphatidylcholine at 37.1% of the total phospholipid present, a value that is comparable to cells from the lung and other tissues. We used similar cells from fetal rat lung and the percent phosphatidylcholine was again 45 to 50%, and 70% of [^3H -CH $_3$]choline was incorporated into the disaturated phosphatidylcholine. Due to the limitations on sample size, we were not able to measure the disaturated form of the phosphatidylcholine in these lung cells. Phosphatidylglycerol, a lipid found in high concentrations in surfactant, was present at 10.7% of the total. Phosphatidylethanolamine, primarily a plasma membrane lipid, was present at 20% of the total. Other phospholipids found in mammalian membranes were present in low amounts. As the Type II cells were serially passaged to four PDL, the phospholipid composition of these cells did not change.

TABLE 2

PHOSPHOLIPID COMPOSITION OF HUMAN FETAL LUNG EPITHELIAL CELLS

Compound	Percent of Total
Phosphatidylcholine	$37.1 \pm 7.6^*$
Phosphatidylglycerol	10.7 ± 2.1
Phosphatidylethanolamine	20.0 ± 1.6
Phosphatidyl (serine + inositol)	13.2 ± 3.2
Sphingomyelin	13.4 ± 1.9
Lysophosphatidylcholine	5.9 ± 3.0

* Mean $\pm$ SEM for seven determinations.

DISCUSSION

We have successfully and reproducibly cultured human fetal lung epithelial cells from cell suspensions derived from human fetal lung (18 to 20 wk gestation). The cells were grown on the surface of the plastic culture dishes without use of a collagen substratum. Morphological examination by light microscopy and examination of the grids by low power electron microscopy (Fig. 4) revealed only a single cell population of Type II pneumocytes. The epithelial cells were joined by desmosomes, had blunted microvilli on their free border, and featured numerous cytoplasmic lamellalike inclusions. These characteristic lamellalike inclusions stained for lipid with Phosphine 3-R and were weakly acid phosphatase positive along their periphery as seen by electron microscopy. We realize they are not the tightly stacked lamellas seen sometimes *in vivo*, however, these structures did not act like phagocytic organelles because the latex beads were not found contained in the organelles. Biochemical lipid analysis of the cultured cells demonstrated a lipid profile consistent with other Type II cell organotypic systems (19). Based on these morphological and biochemical data we interpret the epithelial monolayer cells derived from human fetal lung to represent Type II pulmonary alveolar cells.

The significant features in the successful cultivation of the lung cells, without need of the collagen substratum reported necessary to maintain organotypic cultures, were gentle enzymatic dispersion into single cell suspensions that did not alter their ability to attach to a plastic substratum and selective removal of fibroblasts and 4% CO₂-enriched air atmosphere for adequate growth in a static culture environment. Similar requirements were found for the successful cultivation of monolayers and multilayer cultures of isolated keratinocytes from human neonatal foreskin, adult skin, liver, ovary, and other human organ tissues. The growth pattern and proliferative features of the pulmonary Type II cells were distinct from those of lung fibroblasts. Epithelial cell island growth was not inhibited by fibroblast growth before the separation of the contaminating fibroblasts from the culture dishes.

Ultrastructurally, all stages of development of lamellalike membrane inclusions were found in the cultured cells ranging from collections of membrane-bound residues to complex inclusions consisting of concentric lamellas and of increased electron density. The morphology of these inclu-

sions was consistent with that reported for human Type II alveolar cells in the intact organ (2,12). The lamellalike inclusions of the cultured cells did not resemble phagocytized material and have not been observed as such in other varieties of cells cultured under similar conditions in our laboratory. Further evidence that the cultured epithelial cells were nonphagocytic was demonstrated by their inability to internalize latex beads. Although we recognize that not all phagocytic cells will internalize latex beads, we found that these cells also would not internalize opsonized sheep red blood cells. These results suggest that these cells do not phagocytize. The presence of extensive rough endoplasmic reticulum, as well as intracytoplasmic polysomes, suggested that the cultured lung cells were extremely active. Examination of intact fetal lung tissue before cell separation and culturing revealed that whorl-like or lamellar intracytoplasmic inclusions were not present in any of the lung parenchymal cells at the stage of fetal development used in our culture studies. In addition, most of the epithelial cells in this tissue exhibited only limited rough endoplasmic reticulum and had sparse polysomes suggesting that, at the time of establishment in culture, they had only minimal capacity in protein synthesis. Therefore, it is postulated that isolation-culturing procedures or the culture microenvironment, or both, induced the cytodifferentiation of these cells. An accelerated rate of maturation was seen before with organotypic cultures (2). It is known that Type II alveolar cells can serve as precursor cells for Type I alveolar cells during fetal development, as well as later in life and under certain experimental conditions (20,23). Perhaps the changes resulting in the cultured cells after several subpassages, viz., the assumption of an elongated cell contour, represent a similar type of cytomorphic transition. This parameter, however, has not been examined at this time.

The phospholipid composition of the epithelial cells was performed to gain insight into the nature of the inclusions. They contained a relatively low content of phosphatidylcholine compared to freshly isolated Type II cells from adult animals (7,21). However, it must be pointed out that these cells in our cultures divided. It had not been demonstrated previously that adult Type II cells will divide when maintained in culture (7,24); instead they rapidly deteriorate over 2 to 3 d in culture. We do not mean to imply that the composition is changed by the fact that the

cells divide, but we are only pointing out that the dynamic state of a dividing cell population could contribute to altered phospholipid composition of the population compared to a nonproliferating deteriorating system, (6,23). Several cell lines of Type II origin, which have been derived by culture techniques in the manner described herein, can be used to isolate Type II-like pneumocytes from lung tumors. These cells also can divide and be passaged in culture. The phosphatidylcholine content of these cell lines varies from 40 to 50% (19). The A-549 cell, which is of human origin, contains only 41% phosphatidylcholine (25). Therefore, the phosphatidylcholine content of the Type II cells in this study is not considered to be abnormally low. The low phosphatidylcholine content could be due to the fact that the cells are of fetal origin. However, in consideration of the data from cell lines (19), the low content may be a function of culture conditions. When the Type II cell is maintained in culture, it is more flattened than in vivo. The more flattened shape and possibly the motility of cells in culture may be responsible for the slightly elevated lysophosphatidylcholine content compared to in vivo or freshly isolated cells (7,19,24). The greater content of the lyso species would increase the fluidity of the membranes. In contrast, the present Type II cells contain a high content of phosphatidylglycerol (10.7%) in the range of what is frequently found in surfactant fractions (23).

The phosphatidylglycerol content of adult Type II cells maintained in culture rapidly diminishes within 48 h of culture (7). Type II cell lines contain only negligible levels of phosphatidylglycerol (19). We previously established organotypic cultures of human (2) and fetal rat (15) lung by employing different isolation and culture conditions. Such cultures of rat lung differ from the present cultures in that they contain a greater percentage of phosphatidylcholine and a lower percentage of phosphatidylglycerol. However, the organotypic culture system is considerably different from the present monolayer system. The organotypic system is an aggregate culture system containing both Type II cells and fibroblasts. Secondly, the Type II cells are stationary and do not divide. Also the Type II cells comprise closed spheres where surfactant accumulated, thus permitting the isolation of the surfactant lipoprotein complex from the cultures (14).

The morphology and biochemical features exhibited by these human lung epithelial cells are

commensurate with alveolar Type II cells, i.e. the presence of lamellar bodies and the contents of phosphatidylcholine and phosphatidylglycerol. In these studies we did not fractionate the phosphatidylcholine into the disaturated (DSPC) and unsaturated species. However, DSPC is not a sufficient marker for Type II cells because lung fibroblasts contain it (8) but do not have lamellar bodies, or desmosomes. Although these cells do not fit the criteria of Type II cells as well as freshly isolated adult cells (6-9), it may be unreasonable to expect a dividing cell in culture to resemble totally the Type II cell in vivo, whose growth is normally quiescent. In addition, the freshly isolated adult cells (7-9) were isolated by density gradient procedures. In this manner, there may be a selection for Type II cells that contain an above average content of surfactant phospholipid.

Preliminary report (27) has suggested to us that these Type II cells grown under monolayer culture conditions can be transformed by environmental carcinogens to a transformed phenotype. These transformed cells still exhibited differentiative function while expressing an abnormal phenotype. It is interesting to note that these transformed cells that continue to carry out a differential function have not differentiated terminally. Moreover, it is also interesting to note that the normal Type II cells reported on here, although exhibiting a differentiative function, continue to proliferate in an MEM growth medium supplemented with FBS. This is not the case for bronchogenic keratinocytes. These cells, when cultured in vitro in the presence of FBS-supplemented growth medium, produce keratin and differentiate terminally, (28).

It is felt that normal and consistent growth patterns and cellular proliferation of human cells in monolayer cultures can best be achieved without the inclusion of additional substrata such as collagen, which could add an additional variable factor to the cells' microenvironment. Pure cultures of Type II pulmonary alveolar cells so grown in our laboratory will permit studying the growth and morphologic changes after various conditions modifying the microenvironment and examining changes during the induction of tumorigenesis. For those interested in the developmental process(es) involved in the formation of intracellular lamellar bodies of Type II pneumocytes, the cells grown by the procedure outlined seem ideal.

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